THE EMBO JOURNAL

Cezanne/OTUD7B is a cell cycle-regulated deubiquitinase that antagonizes the degradation of APC/C substrates

Thomas Bonacci, Aussie Suzuki, Gavin D Grant, Natalie Stanley, Jeanette G Cook, Nicholas G Brown & Michael J Emanuele.

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 11th January 2018

Thank you again for submitting your manuscript on Cezanne regulation of APC/C for our consideration. We have now heard back from three expert reviewers, whose comments are copied below for your information. Given their appreciation of the overall interest and potential importance of your findings, we would be in principle happy to consider a revised manuscript further. Nevertheless, it is also apparent that all three referees retain a number of overlapping major concerns that would need to be satisfactorily clarified prior to publication. While several of these issues (such as RNAi controls/rescues, Cezanne/UBE2S co-depletion, or assessing ubiquitination levels of APC/C substrates in cells) should be straightforward to resolve, others (e.g. measuring in vivo degradation kinetics of fluorescent substrates) may require substantial further time and efforts to address.

In light of our policy to consider only a single round of major revision, and since you have indicated being in a competitive situation, I would in this case propose that you provide us with a tentative letter of response to the referee comments already at the start of the revision period, outlining how you would envision addressing each of their concerns, and which revisions would appear to be feasible within a regular revision period. Based on this, we could then determine whether revision of the essential points for EMBO Journal publication would appear realistic, and I could further discuss this work and your revision with our sister journal EMBO Reports, to explore whether a less extensive revision focussing only the most crucial issues would be alternatively suited for rapid publication in their pages.

I should add that in either case, it is our policy that any competing manuscript published elsewhere during this revision will have no negative impact on our final assessment of your revised study, and that we are also open to discussing extensions of our default three-months revision deadline in this case. Additional/more specific information on the preparation of revised manuscripts can be found below.

Thank you again for the opportunity to consider this work for publication, and I look forward to

hearing back from you once you've had the chance to carefully consider the enclosed reviews.

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

REFEREE REPORTS

Referee #1:

The critical mitotic regulators are degraded at specific points in mitosis to drive mitotic progression. Dysregulation of this orderly degradation leads to chromosome missegregation and cytokinesis failure. The anaphase-promoting complex/cyclosome (APC/C) mediates the ubiquitination of important mitotic regulators, such as securin and Cyclin B1, and promotes anaphase onset and mitotic exit. The spindle checkpoint inhibits the premature activation of APC/C bound to Cdc20 until all chromosomes achieve proper attachment to the mitotic spindle. APC/C preferentially assembles K11-linked ubiquitin chains on substrates. Deubiquitination enzymes (DUBs) remove the ubiquitin chains from target proteins and counteract the actions of ubiquitin ligases. Identifying the physiologically relevant DUB that counteracts APC/C in mitosis is important. The current study by Emanuele and coworkers is an important step in that direction.
In this study, Emanuele and coworkers show that Cezanne/OTUD7B (hereafter Cezanne) selectively removes the K11-linked ubiquitin chains of several mitotic regulators. Cezanne binds to Aurora A and Cyclin B1, and slightly delays their degradation. The mRNA and protein levels of Cezanne are both elevated in mitosis, consistent with the proposed mitotic functions in regulating APC/C-Cdc20. Depletion of Cezanne causes chromosome missegregation and reduces cell proliferation. These data suggest that Cezanne negatively regulates APC/C-Cdc20-mediated protein ubiquitination and degradation in mitosis and possibly maintains genomic stability. These findings are significant and should be published in EMBO J. However, the following deficiencies and points need to be addressed experimentally before this paper can be published.
Major points
(1) The authors show that Cezanne binds to Aurora A and Cyclin B in vitro and in unsynchronized, overexpressed cell lysates. However, it is not clear whether Cezanne has substrate specificity towards ubiquitinated Aurora A and cyclin B or generally removes the K11-linked ubiquitin chains of any proteins in mitosis. Is Cezanne in a complex with APC/C-Cdc20 or the proteasome? Is the enzymatic activity of Cezanne regulated? Is it dependent or independent on the spindle checkpoint? The authors should perform immunoprecipitation-mass spectrometry analysis of endogenous Cezanne in mitotic cells, and identify potential interactors/substrates. In addition, they should test if the interaction is regulated in a cell cycle-dependent manner and/or mediated by posttranslational modifications. They should test whether Cezanne isolated from mitosis is more active in cleaving free K11 chains in vitro.
(2) The authors claim that depletion of Cezanne accelerates mitotic progression and causes chromosome missegregation. However, the current data are not strong enough to support this claim. They should monitor mitotic progression using live cell imaging and compare the timing of various mitotic events from nuclear envelope breakdown to mitotic exit. In the same context, they should monitor the degradation kinetics of cyclin B1-GFP or securin-GFP during mitosis in cells with and without Cezanne depletion.
(3) The authors used two different siRNAs for targeting Cezanne. They should provide the Western blot to show knockdown efficiency. Also, they should test if RNAi-resistant Cezanne WT or catalytic dead (C194S) can rescue the defects of mitotic progression in Cezanne-depleted cells, again preferably using live-cell imaging. Given the widespread siRNA off-target effects, the authors may want to knockout Cezanne with CRISPR-Cas9 and confirm the loss-of-function phenotypes.
(4) It is unclear whether the phenotypes caused by Cezanne depletion are due to up-regulation of APC/C-dependent ubiquitination. The authors should co-deplete Ube2S and Cezanne and see if the co-depletion rescues the phenotypes of Cezanne depletion.
Minor points
(1) Statistical analyses should be performed for Figures 1A-B, 1D-E, 3A-D, 4A-B and 5A-B, and Supplementary Figures 1-4.

(2) In Figure 1C, Western blot with the K11 ubiquitin chain-specific antibody would be more informative.
(3) The amount of HA-Cezanne in Supplementary Figure 1B should be presented.
(4) In Supplementary Figure 1D, Western blot analysis should be performed with anti-Cezanne antibodies.
(5) In Supplementary Figure 4B, the labels for siRNAs targeting Cezanne 3, 4, and 3+4 should be changed to 1, 2, and 1+2. Did the authors screen multiple Cezanne siRNAs to identify two that showed the reported phenotypes? If so, this should be stated. For the siRNAs that did not show the phenotypes, did they also deplete Cezanne?

Referee #2:

The work from Bonacci et al. describes a role of the Cezanne/OTUD7B in regulating mitotic progression by antagonizing the degradation of APC/C substrates. The authors show that Cezanne is specific for K11-linked ubiquitin chains, which are formed by APC/C in mitosis. They suggest that by opposing APC/C activity, Cezanne contributes to proper mitotic progression, cell division and proliferation, providing a novel mechanism that regulates mitosis. Most of the biochemical analyses in this manuscript are detailed and well-controlled. However, some studies need improvement to strengthen the manuscript.
Major comments:
1. In Figures 1A and 1B, the authors provide convincing data showing that Cezanne is remarkably specific for K11-linked ubiquitin chains using diubiquitin substrates in agreement with many previous reports. However, as the authors also noticed, there are two reports suggesting that Cezanne also disassembles K48-linked or K63-linked ubiquitin chains (Enesa et al, 2008; Wang et al, 2017). To clarify the specificity of Cezanne toward ubiquitin chains and strengthen the message of this study, the authors need to perform deubiquitination assays to test the effect of Cezanne on K11-linked chains conjugated on known APC/C substrates (as in Figure 3A), but include K63-linked chains conjugated to GβL as a control. Perhaps, rather than adding the ubiquitin ligase and Cezanne at the same time (as in Figure 3A), it would be better to first ubiquitinate substrates and then add Cezanne to evaluate the deubiquitinating activity.

2. The deubiquitinating activity of Cezanne is tested only by in vitro assays. The ubiquitination levels of at least some APC/C substrates need to be shown to prove that the differences observed in protein levels are due to Cezanne mediated de-ubiuquitination. For example, in Figures 3C and 5A, the ubiquitination of some APC/C substrates need to be shown.

Minor issues:

1. Figure 5A: Using time-lapse microscopy can further support the role of Cezanne in the regulation of mitosis progression.

2. Figure 2A: Analysis of the input shows that there is more FoxM1 in the sample co-expressing Myc-FoxM1 and HA-Cezanne than the one expressing Myc-FoxM1 alone (Lane 3 vs. Lane 2), which could lead to the difference seen in the top panel (IP against HA). Perhaps the authors could do the experiment in presence of proteasome inhibitors to obtain similar levels of FoxM1

3. Figure 3A: The robustness of the data in is difficult to determine, as the differences in cyclinB1 ubiquitination levels are small. The authors need to optimize the experimental conditions (see major point #1).

3. Figure 3C and 3D and Supplementary Figure 3B: The Aurora A and Cyclin B western blot should be quantitated.

5. Figure 4A and Supplementary Figure 4A: the effect of different oligoes targeting Cezanne varies. Was the difference due to knocking down efficiency? The effect of different siRNAs (either alone or combined) on Cezanne protein levels needs to be shown by western blot and/or RT-PCR.

6. Supplementary Figure 4B: the labelling of the curves does not match with the western blot data. Is this a typo?

Referee #3:

This work focuses on the protein Cezanne that is a reported DUB with activity towards K11 linked ubiquitin chains but possible also other ubiquitin chain types. The authors firstly confirm that Cezanne is specifically acting on K11 linked ubiquitin chains and this makes them investigate if Cezanne could be a DUB acting on APC/C substrates. The reason for this rationale is that APC/C is known to generate K11-linked chains on substrates by using the E2 enzyme UBE2S. They find that in an extract system addition of Cezanne can reduce APC/C ubiquitination of substrates and this delays their degradation. Indeed Cezanne can directly bind a number of reported APC/C substrates. They explore if depletion of Cezanne by RNAi can affect mitotic fidelity and observe an increase in micronuclei and chromosome alignment errors by fixed cell analysis. Finally they show that Cezanne depleted cells are less efficient in entering S-phase, which requires inhibition of APC/C by Emi1, in line with the idea of Cezanne restricting APC/C activity.
Although several of the experiments are performed at a reasonable level experiments directly linking their biochemical data to their observed cellular data are missing making it very difficult to draw strong conclusions. Given the points outlined below I find the manuscript at a premature stage and not ready for publication in EMBO Journal:

Points:

1) The authors ignore recent work (Wild et al 2016, Garvanska et al 2016 not cited in this manuscript) showing the HCT116 UBE2S KO cells are progressing through mitosis with almost normal kinetics despite complete absence of K11 linked chains. This is consistent with the original work by Garnett et al 2009 that only observed a modest effect of Ube2S RNAi in unperturbed mitosis with no effect of Cyclin B1 degradation kinetics. This is hard to reconcile with the model the authors are proposing here. A simple experiment is to analyze the effect of Cezanne depletion on cells lacking Ube2S: according to the model proposed here Cezanne depletion should have no effects in the absence of Ube2S.

2) If their model is correct many of the cellular phenotypes they report upon RNAi depletion of Cezanne should be due to hyperactive APC/C and should therefore be suppressed by pro-TAME (an APC/C inhibitor).

3) In figure 2 they see an interaction between Cezanne and APC/C substrates using overexpression. It would be worth determining if these interactions occur at endogenous levels of proteins.

4) The in vivo relevance of the experiments in figure 3 are questionable as they are looking at substrate degradation in an extract and adding what appears to be quite large amounts of Cezanne to the extract. Despite a large effect on K11-linked chains in panel A-B the effects on substrate degradation kinetics appears more subtle. The authors should look at substrate degradation kinetics in vivo instead using fluorescent versions of APC/C substrates.
In supplemental figure 3 relating to this figure they see an effect on Aurora A levels in nocodazole which is surprising given that Aurora A is a late APC/C-Cdh1 substrate - there should not be an effect on Aurora A until latter time points! Furthermore I find it strange that the protein levels, particular cyclin B1, in supplemental figure 3 is quite different from that of figure 5A despite being exactly the same experiment.

5) An important point regarding figure 4 is that the experiments in no way links the biochemical data to in vivo function. RNAi rescue with Cezanne WT and catalytic dead mutant is needed and would start linking biochemical and cellular data. Furthermore the experiment suggested in 1) would be very relevant. The in vivo characterization needs to be improved a lot and include the RNAi rescue experiments, time-lapse to look at mitotic duration, in vivo degradation kinetics of substrates, and Cezanne RNAi in a UBE2S null background. Since the SAC is quite a potent inhibitor of the APC/C it is difficult to explain why Cezanne depletion would give such a large increase in alignment errors - increased APC/C activity would not per se lead to alignment defects.

6) I do not see why the data in figure 5 argues for a crucial role of Cezanne in mitotic exit - the Cezanne depleted cells exit just fine and with normal kinetics. Since USP37 has been proposed to be the DUB acting on Cyclin A to promote S-phase entry it would be important to look at cyclin A levels in Cezanne depleted cells.

1st Revision - authors' response 10th May 2018

Response to reviewer comments

MANUSCRIPT OVERVIEW

The Anaphase Promoting Complex/Cyclosome (APC/C) is a large, multi-subunit E3 ubiquitin ligase and core component of the cell cycle machinery. APC/C drives the degradation of myriad substrates, included cyclins, thereby enforcing the oscillations in Cyclin Dependent Kinase (CDK) activity which drive progression through the cell cycle. The APC/C is unique among known E3 ligases in that it targets its substrates for degradation using K11-linked ubiquitin chains. Despite the importance of APC/C in homeostatic cell cycle progression, it was previously unknown if deubiquitinating enzymes (DUBs) antagonize the formation of APC/C formed, K11-linked ubiquitin chains on substrates.
Cezanne is an OTU family DUB, and conflicting reports had previously suggested that it had specificity for different ubiquitin chain topologies. We show here that Cezanne is cell cycle regulated and exquisitely selective for K11-linked ubiquitin chains, consistent with prior reports from the Komander lab. We further demonstrate, using a combination of in vivo and in vitro assays, that Cezanne binds and deubiquitinates APC/C substrates, and controls their degradation in mitosis. Moreover, Cezanne depletion leads to defects in mitotic progression, and these defects are reversed by depletion of the APC/C E2 enzyme that is responsible for the formation of K11-linked chains. We conclude that Cezanne is a cell cycle regulated DUB that antagonizes APC/C substrate degradation.

GENERAL RESPONSE TO ALL REVIEWERS

We would like to thank each of the reviewers for providing thoughtful feedback on this manuscript. All three reviewers provided encouraging comments, highlighting the impact and importance of this study. Each reviewer also offered suggestions on how we could strengthen our overall conclusions. We have taken significant steps to address these comments, through additional experiments and changes to the writing of the manuscript. While our conclusions remain unchanged, they have been strengthened by the additional experiments that you suggested. Perhaps most notable to all three reviewers, we now demonstrate that the mitotic phenotypes associated with Cezanne depletion are reversed by co-depletion of the APC/C E2, UBE2S. Taken together, these changes and additional experiments have improved the overall quality of this study, and we anticipate that you will find this updated draft suitable for publication. Below you will find a point-by-point response to each of the suggestions that you raised. Your prior comments are shown in *italics* and our responses are shown in blue.

REFEREE #1:

The critical mitotic regulators are degraded at specific points in mitosis to drive mitotic progression. Dysregulation of this orderly degradation leads to chromosome missegregation and cytokinesis failure. The anaphase-promoting

complex/cyclosome (APC/C) mediates the ubiquitination of important mitotic regulators, such as securin and Cyclin B1, and promotes anaphase onset and mitotic exit. The spindle checkpoint inhibits the premature activation of APC/C bound to Cdc20 until all chromosomes achieve proper attachment to the mitotic spindle. APC/C preferentially assembles K11-linked ubiquitin chains on substrates. Deubiquitination enzymes (DUBs) remove the ubiquitin chains from target proteins and counteract the actions of ubiquitin ligases. Identifying the physiologically relevant DUB that counteracts APC/C in mitosis is important. The current study by Emanuele and coworkers is an important step in that direction.
In this study, Emanuele and coworkers show that Cezanne/OTUD7B (hereafter Cezanne) selectively removes the K11-linked ubiquitin chains of several mitotic regulators. Cezanne binds to Aurora A and Cyclin B1, and slightly delays their degradation. The mRNA and protein levels of Cezanne are both elevated in mitosis, consistent with the proposed mitotic functions in regulating APC/C-Cdc20. Depletion of Cezanne causes chromosome missegregation and reduces cell proliferation. These data suggest that Cezanne negatively regulates APC/C-Cdc20-mediated protein ubiquitination and degradation in mitosis and possibly maintains genomic stability. These findings are significant and should be published in EMBO J. However, the following deficiencies and points need to be addressed experimentally before this paper can be published.
Major points
(1) The authors show that Cezanne binds to Aurora A and Cyclin B in vitro and in unsynchronized, overexpressed cell lysates. However, it is not clear whether Cezanne has substrate specificity towards ubiquitinated Aurora A and cyclin B or generally removes the K11-linked ubiquitin chains of any proteins in mitosis.
This is a very interesting point. That is, is Cezanne a general DUB for any APC/C substrate, or is it selective for some substrates but not others. Of those we have tested, some, but not all, APC/C substrates are regulated by Cezanne. We have now included these data in the manuscript in Appendix Figure S4. Specifically, we found that Cezanne depletion had no effect on Aurora B or Geminin. Similarly, we now also show that Cezanne cannon deubiquitinate Cyclin B when it is conjugated to other types of ubiquitin chains (K48 and K63 linked). Since Cezanne controls both early and late APC/C substrates, the rules governing how Cezanne designates substrates remains to be determined and represents an important area of future study.

Is Cezanne in a complex with APC/C-Cdc20 or the proteasome?
Since APC/C binding could direct Cezanne towards substrates, we examined Cezanne binding to APC/C. New co-IP data suggests that Cezanne can bind both Cdc20 and Cdh1 (Figure EV2). However, since not all APC/C substrates are controlled by Cezanne, and since it binds to substrates in vitro, independent of APC/C and ubiquitin, this likely represents only one aspect for understanding its role in restraining substrate degradation. We now also demonstrate that Cezanne cannot deubiquitinate model substrates conjugated with K48 and K63 linked chains, even if APC/C is present. Thus, the presence of APC/C is insufficient to allow Cezanne to non-specifically deubiquitinate substrates. Together, this potentially suggests a multi-valency in the interaction between Cezanne and its targets, which we raise in the discussion and hope to address in future studies. Finally, we have no evidence for Cezanne binding to proteasomes.

Is the enzymatic activity of Cezanne regulated? Is it dependent or independent on the spindle checkpoint?

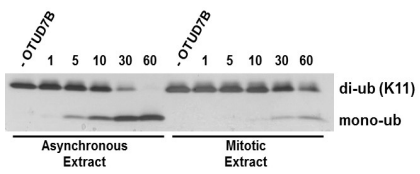


Figure 1. Cezanne/OTUD7B activity appears to be cell cycle regulated. Cezanne was incubated in asynchronous and mitotic cell extracts, recovered and analyzed for its ability to deubiquitinate a K11 di-ubiquitin substrate.

We have tested this hypothesis in two ways. First, we precipitated Cezanne from asynchronous and mitotic cells, and second, we incubated recombinant Cezanne with either asynchronous or mitotic cell extracts. Interestingly, in both assays, incubation with a mitotic cell extract decreases (but does not abolish) Cezanne activity (see Figure 1). Thus, Cezanne activity appears to track that of the APC/C, insofar as its activity is low when the spindle checkpoint is active. This suggests that it might function similarly to a proofreading enzyme. However, we have no insight regarding the mechanisms by which Cezanne is regulated, how its activity is repressed, or how it becomes re-activated. Since we could not envision a way to coherently include these data without raising more questions than we answer, we have decided not to include this in the current manuscript. We hope to pursue this in a future study, where we describe the mechanisms controlling Cezanne activity during cell cycle progression. Notably, we tried to reactivate Cezanne using phosphatases and were unsuccessful in repeated experiments, suggesting that a phosphorylation independent controls its activity.

The authors should perform immunoprecipitation-mass spectrometry analysis of endogenous Cezanne in mitotic cells, and identify potential interactors/substrates.
We agree that a global assessment of Cezanne interactors is an important question. We are planning to develop our own endogenous antibodies, and to leverage these reagents to better understand what and how Cezanne binds substrates, and its mode of regulation during the cell cycle. At this time, we felt this question is beyond the scope of this current study.

In addition, they should test if the interaction is regulated in a cell cycle-dependent manner and/or mediated by posttranslational modifications. They should test whether Cezanne isolated from mitosis is more active in cleaving free K11 chains in vitro.
As mentioned above, we have incubated recombinant Cezanne with asynchronous and synchronized cell extracts and identified a cell cycle regulated activity. We plan to investigate the mechanisms of its cell cycle regulated activity in future studies. Concerning interacting proteins, we show in Figure 2 that the interaction of Cezanne with endogenous Aurora A and Cyclin B is cell cycle regulated.

(2) The authors claim that depletion of Cezanne accelerates mitotic progression and causes chromosome missegregation. However, the current data are not strong enough to support this claim. They should monitor mitotic progression using live cell imaging and compare the timing of various mitotic events from nuclear envelope breakdown to mitotic exit. In the same context, they should monitor the degradation kinetics of cyclin B1-GFP or securin-GFP during mitosis in cells with and without Cezanne depletion.
We agree that this is an excellent experiment, and that in describing our previous data we should have been clearer with the language that we used. Based on this

important suggestion, we have now analyzed mitotic timing in Cezanne depleted cells. Cezanne depletion leads to a small but significant decrease in mitotic duration, now reported in Figure 5. We also monitored Cyclin B degradation in vivo by live cell imaging, as suggested, and consistent with our ability to see it being degraded more quickly by western blot, we see Venus-Cyclin B levels decreasing more rapidly in Cezanne depleted cells. These data are reported in Figure 6.
With regard to the writing, we should have also been more clear, since we think that Cezanne depletion is accelerating the kinetics of APC/C substrate degradation after the initiation of anaphase, and not mitotic progression per se. The text has been modified so that we are more clear about this point. As mentioned above, Cezanne activity is, somewhat surprisingly, low in early mitosis. A potential reason for this would be to allow the burst of APC/C activity needed for the metaphase to anaphase transition.

(3) The authors used two different siRNAs for targeting Cezanne. They should provide the Western blot to show knockdown efficiency. Also, they should test if RNAi-resistant Cezanne WT or catalytic dead (C194S) can rescue the defects of mitotic progression in Cezanne-depleted cells, again preferably using live-cell imaging. Given the widespread siRNA off-target effects, the authors may want to knockout Cezanne with CRISPR-Cas9 and confirm the loss-of-function phenotypes.
We have now shown that Cezanne depletion, which decreases the abundance of APC/C substrates, can be rescued by re-introducing an siRNA resistant version of Cezanne (Figure 4). Given that we had to collaborate with another (very busy) group to do the live cell imaging experiments, we have shown this by immunoblot. These data are consistent with our biochemical data showing that Cezanne binds and deubiquitinates APC/C substrates.

(4) It is unclear whether the phenotypes caused by Cezanne depletion are due to up-regulation of APC/C-dependent ubiquitination. The authors should co-deplete Ube2S and Cezanne and see if the co-depletion rescues the phenotypes of Cezanne depletion.
This is an excellent experiment and was previously a key missing piece of evidence in support of our conclusions. We have now confirmed that Cezanne depletion phenotypes require APC/C in numerous experiments. We show that the mitotic defects after Cezanne depletion are also fully rescued by co-depletion of UBE2S (Figure 5). The same is true for the formation of micronuclei (Figure 5). In addition, the reduction in Cyclin B and Aurora A in Cezanne depleted cells is rescued by UBE2S co-depletion. This provides very strong evidence that these effects are directly related to the role of Cezanne in antagonizing the APC/C.

Minor points
(1) Statistical analyses should be performed for Figures 1A-B, 1D-E, 3A-D, 4A-B and 5A-B, and Supplementary Figures 1-4.
This is a very good point, and this has now been done.

(2) In Figure 1C, Western blot with the K11 ubiquitin chain-specific antibody would be more informative.
We (and others in the field) have faced many challenges in using this antibody. Despite significant efforts, we have been unable to accommodate this suggestion

for purely technical reasons. We hope that better antibodies will become available in the future.

(3) The amount of HA-Cezanne in Supplementary Figure 1B should be presented.
This data has been added.

(4) In Supplementary Figure 1D, Western blot analysis should be performed with anti-Cezanne antibodies.
As stated above, we have had enormous difficulty working with the K11-linkage specific antibody and had no success in repeating that experiment and simultaneously blotting for those chains and Cezanne at the same time. We have communicated with others in the field who have faced similar challenges. Nevertheless, the formation of K11-linked chains in mitosis by APC/C is well-established, and we show the upregulation of Cezanne in multiple cell lines, using orthogonal methods (IF and immunoblotting), with and without synchronization, across many figures and independent experiments, and corroborate cell cycle synchronization by blotting for other cell cycle markers.

(5) In Supplementary Figure 4B, the labels for siRNAs targeting Cezanne 3, 4, and 3+4 should be changed to 1, 2, and 1+2. Did the authors screen multiple Cezanne siRNAs to identify two that showed the reported phenotypes? If so, this should be stated. For the siRNAs that did not show the phenotypes, did they also deplete Cezanne?
We initially tested three siRNA, two of which successfully depleted Cezanne. We therefore focused our experiments on those two reagents. We did not determine if the siRNAs that don't deplete Cezanne give a phenotype. A common off-target for siRNA is Mad2. However, the spindle checkpoint is functional in Cezanne depleted cells (they arrest in nocodazole) indicating that there is no off-target effect on spindle checkpoint regulators. Moreover, we can partially rescue Cezanne phenotypes by expressing an siRNA resistant version. Further, the siRNA results are concordant with the immense amount of biochemical data presented, and is rescued by UBE2S depletion, making a very strong argument for our conclusions. The labeling has been corrected.

REFEREE #2:

The work from Bonacci et al. describes a role of the Cezanne/OTUD7B in regulating mitotic progression by antagonizing the degradation of APC/C substrates. The authors show that Cezanne is specific for K11-linked ubiquitin chains, which are formed by APC/C in mitosis. They suggest that by opposing APC/C activity, Cezanne contributes to proper mitotic progression, cell division and proliferation, providing a novel mechanism that regulates mitosis. Most of the biochemical analyses in this manuscript are detailed and well-controlled. However, some studies need improvement to strengthen the manuscript.
Major comments:
1. In Figures 1A and 1B, the authors provide convincing data showing that Cezanne is remarkably specific for K11-linked ubiquitin chains using diubiquitin substrates in agreement with many previous reports. However, as the authors also noticed, there are two reports suggesting that Cezanne also disassembles K48-linked or K63-linked ubiquitin chains (Enesa et al, 2008; Wang et al, 2017). To clarify the specificity of Cezanne toward ubiquitin chains and strengthen the

message of this study, the authors need to perform deubiquitination assays to test the effect of Cezanne on K11-linked chains conjugated on known APC/C substrates (as in Figure 3A), but include K63-linked chains conjugated to GβL as a control.
This is a very important point and we completely agree that clarifying the specificity of Cezanne is extremely important. This is especially true in light of recent conflicting reports which, on one hand, reported the molecular basis for Cezanne specificity for K11-chains using recombinant enzyme, and on the other, suggested that Cezanne could disassemble K63-linked chains in vivo. Notably, there is a recent report that post-translational modification of DUBs in vivo can alter their specificity for ubiquitin linkages. Nevertheless, our results, in the previous submission and through further experiments here, are in strong agreement with those of Komander and colleagues, who demonstrated Cezanne specificity for K11-linked chains.
We sought to address this question more thoroughly, based on your suggestion. However, we have no experience working with GβL or the enzymes involved in its ubiquitination. Instead, we devised an alternative experiment, and one which is specifically relevant to our studies. Using non-physiological E2s at high concentrations, we ubiquitinated cyclin B and securin with either K11, K48 or K63 linked ubiquitin chains and determined the ability of Cezanne to deubiquitinate them in vitro. Importantly, Cezanne can bind substrates in vitro, independent of their ubiquitination. However, our new data show that Cezanne is unable to deubiquitinate K48 and K63 linked chains formed on Cyclin B or Securin (see Figures 3 and EV3). These data strongly support a role for Cezanne in *specifically* antagonizing K11 linked chains.

Perhaps, rather than adding the ubiquitin ligase and Cezanne at the same time (as in Figure 3A), it would be better to first ubiquitinate substrates and then add Cezanne to evaluate the deubiquitinating activity.
This is a very good point and suggestion. Indeed, Figure 3A (which is now 3C), has both the APC/C isolated by IP from HeLaS3 cells and recombinant Cezanne at the same time. However, in the experiments where Cyclin B and Securin were ubiquitinated using recombinant APC/C, Cdc34 or Ubc13+UEV1, the ubiquitination reactions were stopped by addition of EDTA. Therefore, the deubiquitination by Cezanne was analyzed after substrate ubiquitination as you suggested. We changed the text accordingly in order to make it more clear for the reader.

2. The deubiquitinating activity of Cezanne is tested only by in vitro assays. The ubiquitination levels of at least some APC/C substrates need to be shown to prove that the differences observed in protein levels are due to Cezanne mediated de-ubiuquitination. For example, in Figures 3C and 5A, the ubiquitination of some APC/C substrates need to be shown.
This is a very good suggestion. To address this question we took advantage of assays that measure APC/C substrate ubiquitination under physiological conditions, but that alleviate concerns over alterations in cell cycle progression which could be introduced by our manipulations. Using a HeLaS3 cell G1-phase extract system, popularized by the Kirchner and Rape labs, we show that Aurora A ubiquitination is directly affected by Cezanne (but not a catalytically dead mutant version). These data, together with biochemical binding and deubiquitination data, as well as in

vivo degradation experiments and phenotypic assays, strongly support the conclusion that Cezanne antagonizes the APC/C by deubiquitinating APC/C substrates.

Minor issues:
1. Figure 5A: Using time-lapse microscopy can further support the role of Cezanne in the regulation of mitosis progression.
This point was raised by all reviewers. We therefore examined mitotic timing using GFP-H2B U2OS cells, as well as substrate degradation using a fluorescently tagged Cyclin B (Venus-Cyclin B). Both results support our previous conclusions, that mitosis is accelerated in Cezanne depleted cells and that substrates are degraded more rapidly after metaphase. These data are now included in Figure 5 and 6.

2. Figure 2A: Analysis of the input shows that there is more FoxM1 in the sample co-expressing Myc-FoxM1 and HA-Cezanne than the one expressing Myc-FoxM1 alone (Lane 3 vs. Lane 2), which could lead to the difference seen in the top panel (IP against HA). Perhaps the authors could do the experiment in presence of proteasome inhibitors to obtain similar levels of FoxM1
Figure 2A was repeated using MG132 following your suggestion so that the levels of FoxM1 are better normalized between IP conditions. Similar to the previous result, we found a strong interaction between FoxM1 and Cezanne.

3. Figure 3A: The robustness of the data in is difficult to determine, as the differences in cyclinB1 ubiquitination levels are small. The authors need to optimize the experimental conditions (see major point #1).
We have now included an additional experiment in Appendix Figure S2 where the high molecular weight forms of Cyclin B are almost entirely gone when Cezanne is present in the reaction. However, we decided to leave the initial experiment (Figure 3C) in the manuscript as these differences are probably more representative of the equilibrium between rounds of ubiquitination by the APC/C and deubiquitination by Cezanne, when both are present and active at the same time. Along with our experiments using recombinant APC/C, where K11 ubiquitin chains are removed by Cezanne, it is clear that Cezanne can antagonizes APC/C ubiquitination.

3. Figure 3C and 3D and Supplementary Figure 3B: The Aurora A and Cyclin B western blot should be quantitated.
Most of this blotting was done using film, and while this can be quantified to some degree, the data reported is semi-quantitative (at best) and can easily mis-interpreted. Since we show these results with two different substrates, and because they corroborate a significant amount of additional data, we hesitate to quantify this result, which is inherently qualitative (that it, Cezanne restrains degradation). Nevertheless, if this is a requirement of yours for publication, we would obviously do so.

5. Figure 4A and Supplementary Figure 4A: the effect of different oligoes targeting Cezanne varies. Was the difference due to knocking down efficiency? The effect of different siRNAs (either alone or combined) on Cezanne protein levels needs to be shown by western blot and/or RT-PCR.
We initially identified two siRNAs that deplete Cezanne and used these for our studies. We show their knockdown efficiency in Appendix Figure S5. This point is

clarified in the manuscript. Both oligos, alone and in combination, significantly reduce Cezanne proteins levels, so it is possible that these slight differences are due to differences in the degree of knockdown that is beyond our current level of detection.

6. Supplementary Figure 4B: the labelling of the curves does not match with the western blot data. Is this a typo?
This is a typo, thank you for pointing this out. This has been corrected.

REFEREE #3:

This work focuses on the protein Cezanne that is a reported DUB with activity towards K11 linked ubiquitin chains but possible also other ubiquitin chain types. The authors firstly confirm that Cezanne is specifically acting on K11 linked ubiquitin chains and this makes them investigate if Cezanne could be a DUB acting on APC/C substrates. The reason for this rationale is that APC/C is known to generate K11-linked chains on substrates by using the E2 enzyme UBE2S. They find that in an extract system addition of Cezanne can reduce APC/C ubiquitination of substrates and this delays their degradation. Indeed Cezanne can directly bind a number of reported APC/C substrates. They explore if depletion of Cezanne by RNAi can affect mitotic fidelity and observe an increase in micronuclei and chromosome alignment errors by fixed cell analysis. Finally they show that Cezanne depleted cells are less efficient in entering S-phase, which requires inhibition of APC/C by Emi1, in line with the idea of Cezanne restricting APC/C activity.
Although several of the experiments are performed at a reasonable level experiments directly linking their biochemical data to their observed cellular data are missing making it very difficult to draw strong conclusions. Given the points outlined below I find the manuscript at a premature stage and not ready for publication in EMBO Journal:
Points:
1) The authors ignore recent work (Wild et al 2016, Garvanska et al 2016 not cited in this manuscript) showing the HCT116 UBE2S KO cells are progressing through mitosis with almost normal kinetics despite complete absence of K11 linked chains. This is consistent with the original work by Garnett et al 2009 that only observed a modest effect of Ube2S RNAi in unperturbed mitosis with no effect of Cyclin B1 degradation kinetics. This is hard to reconcile with the model the authors are proposing here. A simple experiment is to analyze the effect of Cezanne depletion on cells lacking Ube2S: according to the model proposed here Cezanne depletion should have no effects in the absence of Ube2S.
You bring up several interesting and important points related to the dynamics and contributions of various E2 enzymes in APC/C regulation, mitotic progression and mitotic exit. We are well aware of these studies, which demonstrate that cells can live without UBE2S, UBE2C, or loss of both in combination. These two important papers indicate that the APC/C is flexible in using others E2s if its preferred ones are unavailable. In addition, and more importantly, they show that interfering with APC/C activation and substrate degradation has only a minimal effect on mitotic progression. We apologize for not citing these papers and have now cited the *Wild et al* paper.

Nevertheless, there is a wide agreement that UBE2C and UBE2S represent the physiological E2s for the APC/C. Our result after Cezanne depletion suggests a role in mitotic progression. However, we do not see our result as contradicting these prior studies. Since Cezanne antagonizes APC/C, a similar experiment would be to test UBE2S overexpression.
Based on your excellent suggestion, and to further strengthen the conclusion that the Cezanne depletion phenotypes are due to its role in antagonizing APC/C, we co-depleted Cezanne with UBE2S. Significantly, this completely reversed the formation of micronuclei, as well as the mitotic defects observed in Cezanne depleted cells. This, together with our immense amount of biochemical data, argues very strongly that the defects in mitosis which we observed in Cezanne depleted cells are dependent on APC/C. These new results are now reported in Figure 5.

2) If their model is correct many of the cellular phenotypes they report upon RNAi depletion of Cezanne should be due to hyperactive APC/C and should therefore be suppressed by pro-TAME (an APC/C inhibitor).
To address the concern that the effects we are observing in cellular phenotypes are a result of increased APC/C output we have performed several experiments. First, we examined the ubiquitination of APC/C substrates under physiological conditions and find that Cezanne counteracts ubiquitination (Figure 3). Second, we show that Cezanne depletion decreases the abundance of the APC/C substrates Aurora A and Cyclin B, which can be rescued by co-depletion of UBE2S (Figure 4). Finally, we demonstrate that the mitotic defects and micronuclei formation in Cezanne depleted cells is dependent on UBE2S (Figure 5).

3) In figure 2 they see an interaction between Cezanne and APC/C substrates using overexpression. It would be worth determining if these interactions occur at endogenous levels of proteins.
We agree that this is an excellent experiment. Unfortunately, despite a lot of effort, we were unable to perform endogenous IPs between these proteins. We think this is because their interaction is transient during the cell cycle, and because we lack endogenous IP-quality antibodies for the proteins being tested. However, the data presented in Figure 2E shows that both endogenous Aurora A and Cyclin B can be pulled down by a recombinant version of Cezanne, specifically in mitosis. This is in addition to co-IP binding following ectopic overexpression of both, binding in vitro using purified proteins, and in vitro deubiquitination of bona fide APC/C substrates. We hope that these biochemical data, and the showing the Cezanne regulates their degradation and ubiquitination, is convincing enough without showing fully endogenous co-IP experiments

4) The in vivo relevance of the experiments in figure 3 are questionable as they are looking at substrate degradation in an extract and adding what appears to be quite large amounts of Cezanne to the extract. Despite a large effect on K11-linked chains in panel A-B the effects on substrate degradation kinetics appears more subtle. The authors should look at substrate degradation kinetics in vivo instead using fluorescent versions of APC/C substrates.
This is a very good suggestion and was raised by multiple reviewers. Consistent with biochemical data showing accelerated APC/C substrate degradation at mitotic exit in Cezanne depleted cells, we now show that Venus-Cyclin B is also degraded

more rapidly in Cezanne depleted cells going through an unperturbed mitosis. These data are reported in Figure 5.

In supplemental figure 3 relating to this figure they see an effect on Aurora A levels in nocodazole which is surprising given that Aurora A is a late APC/C-Cdh1 substrate - there should not be an effect on Aurora A until latter time points! Furthermore I find it strange that the protein levels, particular cyclin B1, in supplemental figure 3 is quite different from that of figure 5A despite being exactly the same experiment.

We apologize for the confusion. The experiments in Appendix Figure S3 and 5A were in fact from different experiments. The supplemental figure was looking at stability following the addition of cycloheximide to mitotic cells, as noted in the figure legend and in the marking of the figure itself. Alternatively, in Figure 5A we are looking at cells progressing out of mitosis after a mitotic block and release in the microtubule depolymerizing drug nocodazole. In Appendix Figure S3, there is indeed less Aurora A at the zero time point in Cezanne depleted cells compared to controls. This is evident in the zero time point of the experiment in Figure 5, indicating that this is a consistent result. While the reason for this remains unknown, we would predict that although Aurora A is normally a late mitotic substrate, this has only ever been analyzed in cells with active Cezanne. As was shown by Catherine Lindon's lab (*Min et al, MBOC, 2015*), Aurora A is ubiquitinated with K11-linked chains at mitotic exit. Together, these data strongly suggest that Cezanne antagonizes Aurora A ubiquitination, and might contribute to making it a late APC/C substrate. We discuss the possibility that Cezanne is implicated in the ordering of substrate degradation in the discussion. This is an important, unresolved question in the APC/C field and one we hope to address in detail in the future.

5) An important point regarding figure 4 is that the experiments in no way links the biochemical data to in vivo function. RNAi rescue with Cezanne WT and catalytic dead mutant is needed and would start linking biochemical and cellular data. Furthermore the experiment suggested in 1) would be very relevant. The in vivo characterization needs to be improved a lot and include the RNAi rescue experiments, time-lapse to look at mitotic duration, in vivo degradation kinetics of substrates, and Cezanne RNAi in a UBE2S null background. Since the SAC is quite a potent inhibitor of the APC/C it is difficult to explain why Cezanne depletion would give such a large increase in alignment errors - increased APC/C activity would not per se lead to alignment defects.

You bring up several interesting and important points. As is the case when comparing biochemical and cell biological data, these results often can only provide a correlation. To help address these concerns we have performed several additional experiments. First, and most importantly, we now demonstrate that the mitotic defects and micronuclei formation observed in Cezanne depleted cells is completely reversed by co-depletion of UBE2S. These new results, combined with the biochemical data showing that Cezanne binds, deubiquitinates and controls the stability of APC/C substrates, provides a very strong argument that the Cezanne phenotypes are due to its antagonism of APC/C.

In addition, we now show that the effect of Cezanne depletion on APC/C substrate abundance can be rescued by the re-expression of an siRNA resistant version. We measured mitotic duration, as you suggested, and show that Cezanne depleted cells

have a small, but significant decrease in the time from NEB to anaphase. Finally, we examined Venus-Cyclin B degradation in control and Cezanne depleted cells, and this result recapitulates the immunoblot data already presented. Together, these results support the conclusion that Cezanne antagonizes APC/C substrate ubiquitination.
Finally, it is established that accelerating mitotic progression can lead to defects in mitotic progression. In yeast and human cells, loss of the spindle checkpoint proteins leads to an increased rate of chromosome mis-segregation. Likewise, depletion of checkpoint proteins, which shortens mitosis, increases chromosome segregation errors. In addition, it is clear from experiment performed in the Kirschner, Pines and King labs that there is residual APC/C activity in early mitosis, and this contributes to spindle checkpoint inactivation. Furthermore, impairing the activation of APC/C using proTAME was shown by several groups to improve chromosome segregation fidelity.

6) I do not see why the data in figure 5 argues for a crucial role of Cezanne in mitotic exit - the Cezanne depleted cells exit just fine and with normal kinetics. Since USP37 has been proposed to be the DUB acting on Cyclin A to promote S-phase entry it would be important to look at cyclin A levels in Cezanne depleted cells.
We apologize for not using more clear language. What we should have more clearly articulated is that these data point to an important role for Cezanne in mitotic progression and the degradation of APC/C substrate during mitotic exit. We have more carefully worded these statements so as not to give the impression that we are over-interpreting our results.
With respect to Cyclin A, the result of Cezanne depletion on Cyclin A is less clear, and it appears Cyclin A is not regulated by Cezanne. This makes sense since cells destroys it very early, and if Cyclin A was controlled by Cezanne, this could prevent Cyclin A degradation in early mitosis. However, these results are more preliminary and we therefore chose not to include them. In the future, we plan to test an extensive list of substrates, using in vivo and in vitro assays, to gain a better sense for how Cezanne selects substrates.

2nd Editorial Decision 13th June 2018

Thank you again for submitting your revised manuscript for our consideration. It has now been seen once more by the original reviewers, and I am happy to inform you that all three of them are generally satisfied with the revisions and improvements to the paper and have no more principle objections toward publication in The EMBO Journal. Referee 3 still retains a few specific criticisms, most of which could in my view be sufficiently addressed by responding in writing and acknowledging in a modified manuscript text. I do however agree with this reviewer's third criticism, and feel that the rescue with RNAi-insensitive Cezanne shown in Fig 4E would indeed need to be supported by more convincing data/stronger effects on substrate level rescue (maybe by achieving stronger Cezanne re-expression?), ideally side by side with reexpression of a catalytically inactive Cezanne version as previously requested in the first set of reports. In addition, the paper would clearly be further strengthened if you should be able to assess rescue by Cezanne reexpression, as well as Ube2S single-depletion effects, also in one of the cell biological assays in Figure 5; but I do understand that this may be more demanding because of the need to collaborate.

I am therefore returning the paper to you for a final round of minor revision, hoping that you will be able to take care of the remaining scientific and editorial issues in a straightforward manner and that upon resubmission, we should be able to swiftly proceed with acceptance and publication of the study. Should you have any additional questions in this regard, please do not hesitate to contact me.

REFEREE REPORTS

Referee #1:

The authors have addressed most of my concerns. Even though they could not address the cell cycle regulation of the enzyme, I agree with their argument that this aspect can be probed in a separate future study. The addition of the Ube2S/Cezanne double depletion data greatly strengthens their argument. I can support the publication of this study in EMBO J.

Referee #2:

The authors have thoroughly addressed my concerns and appear to have satisfactorily addressed the concerns of the other reviewers.

Referee #3:

The authors should be acknowledge for performing a good revision and for doing the Ube2S depletion experiments. I do have several issues still also with the overall logic of the paper.
1) There is no data to substantiate the interactions of Cezanne at the endogenous levels which would clearly have strengthen the story. At minimum this should be stated at the end of the section.
2) As Cezanne is being degraded as cells exit it seems very strange to look at its role in controlling APC/C-Cdh1 in G1 and the authors do not really justify this. One would ideally have looked in a mitotic extract running on APC/C-Cdc20.
3) The rescue of Cezanne RNAi in Fig 4E is not very convincing and should at least also have been done for one of the phenotypic readouts in Figure 5.
4) The experiments in Figure 5 where they rescue with Ube2S RNAi - for all these one should have done Ube2S alone otherwise on cannot extract much from these experiments.

Minor points:
Page 11: They see no effect on mitotic exit when they deplete Cezanne which seems add odds with later statements on faster cyclin B1 degradation - cells should exit faster.
Page 11/Figure 4: Seeing an effect on Aurora A levels when Cezanne is depleted is very difficult.

2nd Revision - authors' response — 14th June 2018

Response to reviewer comments

Overview

We would like to thank the reviewers for carefully considering our revised manuscript. We were happy to see that all three reviewers appreciated the effort that we made to address the comments made during the first round of reviews.

We were strongly encouraged by the fact that reviewers one and two were in full support of publication of our revised manuscript in EMBO J. Reviewer 3 had a few remaining concerns, which we address in a point-by-point response below. Our responses to reviewer comments are in blue.

- Referee #1

The authors have addressed most of my concerns. Even though they could not address the cell cycle regulation of the enzyme, I agree with their argument that this aspect can be probed in a separate future study. The addition of the Ube2S/Cezanne double depletion data greatly strengthens their argument. I can support the publication of this study in EMBO J.

Thank you for the time and energy you spent in reviewing and considering the manuscript. We look forward to exploring the cell cycle regulated activity of Cezanne in future studies.

- Referee #2

The authors have thoroughly addressed my concerns and appear to have satisfactorily addressed the concerns of the other reviewers.

We appreciate your time in considering our manuscript and are happy to hear that we were able to address all of the concerns raised in the initial review.

- Referee #3

The authors should be acknowledge for performing a good revision and for doing the Ube2S depletion experiments. I do have several issues still also with the overall logic of the paper.

1) There is no data to substantiate the interactions of Cezanne at the endogenous levels which would clearly have strengthen the story. At minimum this should be stated at the end of the section.

First, thank you for evaluating our revised manuscript, providing thoughtful and carefully considered feedback, and for acknowledging the tremendous amount of work that went into the revised draft of the manuscript.

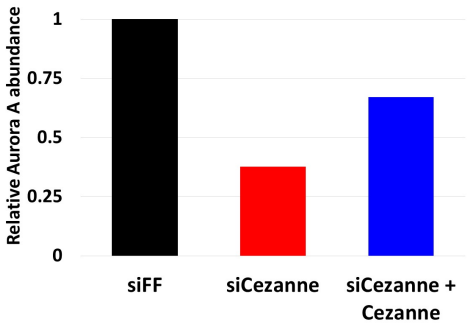
In this study, we show that Cezanne binds established APC/C substrates in vitro using recombinant proteins, and in cells by ectopic expression followed by coIP. We also performed a semi-endogenous pull downs of Aurora A and Cyclin B from U2OS extracts, using recombinant Cezanne as a bait, which somewhat compensates for the lack of endogenous coIP. Further, we showed that Cezanne deubiquitinates substrates, both in vitro and in physiological settings, and regulates their stability in vivo. Nevertheless, we were unable to detect an interaction between endogenous proteins. We suspect this is because our antibodies do not work well for endogenous IP, and because the interactions are transient and cell

cycle regulated. We have now stated clearly that we were unable to detect endogenous interactions (pg 17).

2) As Cezanne is being degraded as cells exit it seems very strange to look at its role in controlling APC/C-Cdh1 in G1 and the authors do not really justify this. One would ideally have looked in a mitotic extract running on APC/C-Cdc20.

You correctly point out that Cezanne is degraded at mitotic exit. However, Cezanne likely does not regulate APC/C per se, but rather controls its substrates. We choose to analyze substrate stability in G1 extracts, as opposed to mitotic cell extracts, for the following reasons. Since the spindle assembly checkpoint is active in mitotic extracts, APC/C activity is very low. Whereas, in G1 extracts, the APC/C is maximally activated. This can be overcome in mitotic extracts by adding proteins that inactivate the checkpoint, such as p31/comet. However, it is unknown how the addition of p31/comet, or other proteins (TRIP13, etc), might affect Cezanne activity and potentially complicate the interpretation of these experiments. Thus, by using a G1 extract, we have as close to a purely physiological system as is possible where APC/C is maximally active.

3) The rescue of Cezanne RNAi in Fig 4E is not very convincing and should at least also have been done for one of the phenotypic readouts in Figure 5.



Re-expression of RNAi-resistant Cezanne partially rescues the abundance of Aurora A. Semi-quantitative analysis of Aurora A levels showed that Cezanne depletion reduced Aurora A levels to ~37% of siFF control. Re-expression of RNAi-resistant Cezanne in depleted cells partially rescued Aurora A levels, increasing it to 67% of controls.

In Figure 4E we show that Cezanne knockdown reduces the abundance of Aurora A, and that this is partially rescued by the re-expression of an RNAi-resistant form of Cezanne. We have performed a semi-quantitative analysis of the gel bands, which showed that Cezanne re-expression after RNAi, despite being below endogenous levels, provided an ~50% rescue of Aurora A protein levels. Since Cezanne depletion impairs cell cycle progression, knock-down and rescue experiments were technically challenging. We too would have preferred a 100% rescue. However, these data are fully consistent with the rest of the data in the paper, which includes significant biochemical evidence, which altogether indicates that Cezanne antagonizes APC/C substrate ubiquitination.

4) The experiments in Figure 5 where they rescue with Ube2S RNAi - for all these one should have done Ube2S alone otherwise on cannot extract much from these experiments.

We had previously performed the Ube2S alone knockdown and monitored micronuclei formation and have now included this data in Figure 5.

Minor points:
Page 11: They see no effect on mitotic exit when they deplete Cezanne which seems add odds with later statements on faster cyclin B1 degradation - cells should exit faster.

We apologize for this not being clearer. Cezanne depletion slightly accelerates progression through mitosis (nuclear envelope breakdown to anaphase), and this is reversed by co-depletion of Ube2S. In addition, Cyclin B degradation is accelerated, as measured by immunoblot and live imaging. Additional substrates analyzed by immunoblot show a similar phenotype. We have made minor modifications to the text (pgs 13-14) to further clarify our description of these results.

Page 11/Figure 4: Seeing an effect on Aurora A levels when Cezanne is depleted is very difficult.

These experiments were performed in asynchronous cells. Even though Cezanne regulates Aurora A in and around mitosis, there is still a clear decrease in Aurora A levels following Cezanne depletion, although admittedly, this is not an all or nothing difference in these experiments. However, these data are consistent with the biochemical data in Figure 3 and data in synchronized cells in Figure 6.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ê

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Michael Emanuele
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2017-98701

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ê (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	(This is the response corresponding to box plot 1): In Figure (5), the sample sizes were 150 for each of the three conditions. (This is the response corresponding to box plot 2): In Figure (6), the sample sizes were 30 for each of the two conditions. In both cases, these sample sizes are adequate for the performed two sample Wilxocon tests.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	The analysis of Venus-Cyclin B degradation in mitosis was performed in a blinded fashion. That is, the person performing the analysis was unaware of which sample wells corresponded to which treatment conditions. See Fig 5.
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes. One of our authors is a PhD level scientist with a degree in mathematics and she determined and carried out appropriate statistical tests.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	For the comparisons shown in Figures (5) and (6), the data were not normally distributed. For this reason, we did not perform a two sample t-test. Instead, we used the Wilcoxon test, a nonparametric method that does not require a normality assumption. Each Wilcoxon test was a two-sided test and produced very significant p-values (p<<0.01)

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http://biomodels.net/	Biomodels Database
http://biomodels.net/miriam/	MIRIAM Guidelines
http://jjj.biochem.sun.ac.za	JWS Online
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html	Biosecurity Documents from NIH
http://www.selectagents.gov/	List of Select Agents

Is there an estimate of variation within each group of data?	Boxplot 1 in Figure 5: siLUC(mean=36.86, sd=9.91), siCez(mean=32.3, sd=7.10), siCez and siUBE (mean=39.66, sd=9.68) Boxplot 2 in Figure 6: siCez(mean=37.2,sd=7.62), siFF(mean=62.8,sd=18.62)
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	We purchased antibodies commerically and the catalog numbers are provided. For Cezanne antibodies, which is the major focus of this work, we confirmed their specificity using knockdown experiments with siRNA.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Cell lines were obtained from ATCC or the UNC Tissue Culture Facility, which procures and distributes cell lines that were purchased from trusted commercial vendors (e.g ATCC).

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No.